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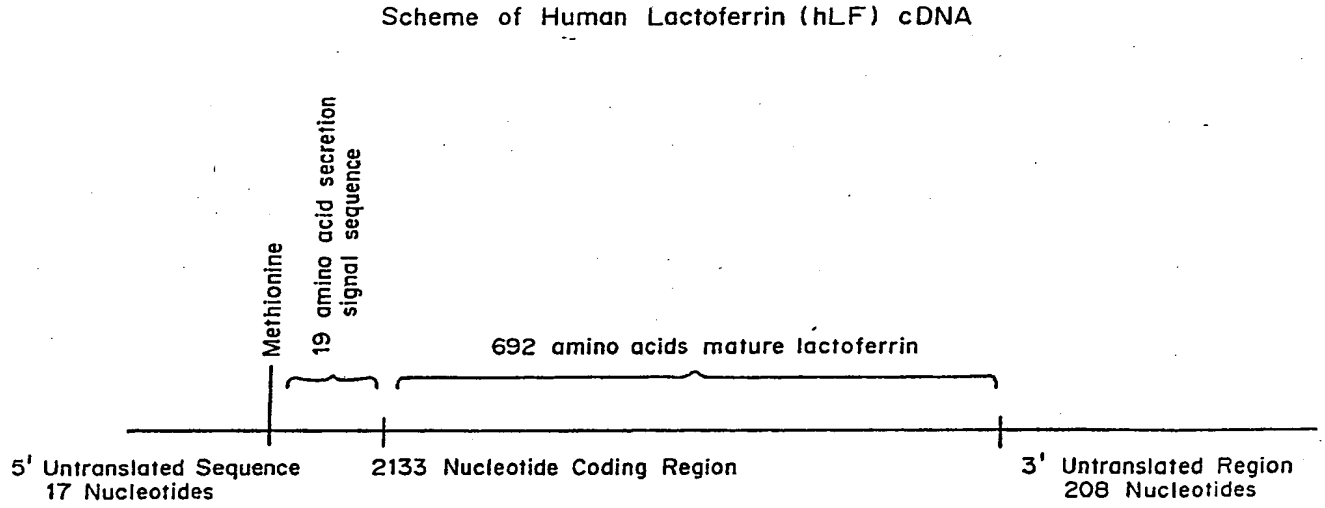

Complete Human Lactoferrin : 711 amino acids
Mature Human Lactoferrin : 692 amino acids

(57) Abstract

The verified cDNA sequence for human lactoferrin (hLF) protein has been discovered. The cDNA sequence can be used to prepare recombinant human lactoferrin for therapeutic and nutritional applications. Regions of the cDNA such as the Fe binding sites can be used to make an hLF polypeptide product.

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HUMAN LACTOFERRIN cDNA SEQUENCE

BACKGROUND OF THE INVENTION

Lactoferrin is an iron-binding glycoprotein found in milk and other secretions and body fluids. It is one of a number of iron binding proteins, sometimes referred to as transferrins, and is involved in iron binding and delivery in mammals.

Lactoferrin has been implicated as a factor in resistance against enteritis infections in suckled newborns. These bacteriocidal/bacteriostatic actions are considered to be due at least in part to the iron binding properties of lactoferrin. Lactoferrin decreases the iron availability to iron requiring microorganisms and thereby interferes with their growth and reproduction. Lactoferrin is also considered to have antiviral properties and to have other potential therapeutic applications.

Human lactoferrin (hLF) has a high affinity for iron and two Fe^{3+} cations can be found per molecule. The complete hLF protein has been subjected to amino acid sequencing and is reported to have 703 amino acids. There are two glycosylation sites. Metz-Boutigue et al; "Human Lactotransferrin: Amino Acid Sequence and Structural Comparisons With Other Transferrins", Eur. J. Biochem., vol. 145, pp. 659-676 (1984). Anderson et al; "Structure of Human Lactoferrin at 3.2 - A Resolution", Proc. Nat'l. Acad. Sci. USA, vol. 84, pp. 1769-1773 (April 1987).

In other studies, a cloned cDNA probe for amino acids 428 to 703 of the lactoferrin protein was isolated. The cDNA sequence was in general agreement with the earlier analysis of the amino acid sequence of the protein. Rado, et al; "Isolation of Lactoferrin cDNA From a Human Myeloid

Library and Expression of mRNA During Normal and Leukemic Myelopoiesis", Blood, vol. 79, no. 4, pp. 989-993 (Oct. 1987). The probe was reported to encompass approximately 40% of the coding region and the 3' terminus. The full confirmed cDNA sequence has not been reported.

SUMMARY OF THE INVENTION

The invention is the verified cDNA sequence for human lactoferrin which includes an open reading frame of 2133 nucleotides coding for a protein of 711 amino acids. These 711 amino acids include 19 amino acids corresponding to a secretion signal peptide sequence followed by 692 amino acids of mature human lactoferrin. The cDNA sequence and deduced amino acid sequence differ from the previously published data. The confirmation of the cDNA sequence and the deduced amino acid have been proven by multiple confirmation procedures.

These are:

1. Multiple sequence analyses.
2. Comparison of the amino acid sequence deduced from the cDNA with that of human LF generated by conventional amino acid sequencing of hLF isolated from milk. The unique cDNA sequence which encodes the human lactoferrin protein has a variety of applications as known and indicated in the literature.
3. Transcription and translation of hLF protein from the cDNA with positive identification using an anti hLF antibody.

The present invention of the cDNA sequence can be used to prepare recombinant human lactoferrin, thus making available a source of human protein for therapeutic and nutritional applications. The confirmed cDNA of this invention can be used in an appropriate cloning vehicle to replicate the cDNA sequence. Also, the cDNA can be incorporated into a vector system for human lactoferrin

expression. This invention is not limited to any particular uses of the cDNA. The recombinant hLF being a human protein derived by recombinant techniques can be used in a variety of applications. The gene can be transferred to mammalian systems such as cows and other agriculturally important animals and expressed in milk. The incorporation of a human lactoferrin and expression in the milk of animals can combat an iron deficiency typical in piglets. The inclusion of the human lactoferrin gene with expression should improve an animal's disease resistance to bacterial and viral infection. The tissue specific expression of human lactoferrin in mammary glands, for instance, would impart the bacteriocidal and viracidal benefit of the expressed gene to young feeding on the milk and would provide a production means for the secreted protein for therapeutic use.

The gene can be placed in the appropriate cloning vector for the production of hLF. The hLF produced by recombinant methods can be used in a variety of products including human or animal foods, as therapeutic additives to enhance iron transport and delivery, and for the viracidal and bacteriocidal qualities, as additives for eye-drops, contact lens and other eye care solutions, topical skin care products, ear drops, mouthwashes, chewing gum and toothpaste. The recombinant hLF would provide a safe, naturally occurring product which can be topically applied as well as ingested safely.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic drawing of the hLF cDNA including the locations of the 5' untranslated region, the secretion peptide signal sequence, mature lactoferrin and 3' untranslated region.

Fig. 2 is the cDNA sequence with deduced amino acids for the human lactoferrin protein and signal peptide

sequence.

Fig. 3 is a schematic representation of an autoradiograph of recombinant human lactoferrin protein expressed from the complete cDNA.

Fig. 4 is a schematic representation of an autoradiograph of the results of in vitro translation of a 2,140 bp human lactoferrin sequence and hLF protein in reticulocyte lysates.

DETAILED DESCRIPTION OF THE INVENTION

The full length cDNA encoding human lactoferrin (hLF) has been isolated, and the analysis has been completed. The cDNA sequence has been confirmed as human lactoferrin cDNA by comparison of the deduced amino acid sequence with the published amino acid sequence of human LF. The expression of lactoferrin was observed in an eucaryotic expression system from the cDNA and a plasmid vector. The presence of lactoferrin was confirmed by standard Western immunoblot analysis using anti-human lactoferrin antibodies and relative molecular mass measurement.

Fig. 1 is a schematic of the lactoferrin cDNA. The sequence can generally be described as an initial 5' untranslated region, 17 nucleotides in length. The next portion is 57 nucleotides which codes for the 19 amino acid secretion signal peptide starting with methionine. The next sequence of the cDNA codes for the mature human lactoferrin protein of 692 amino acids followed by the 3' untranslated region of 208 nucleotides which ends the cDNA. The complete sequence is 2,358 nucleotides in length. The hLF protein contains glycosylation sites. The hLF protein with secretion signal sequence has an expected molecular mass of 78,403 daltons and the mature hLF is 76,386 daltons without added carbohydrate from glycosylation.

Fig. 2 is the cDNA sequence with the deduced amino acids for the secretion signal peptide and the mature human lactoferrin protein. The numbers on Fig. 2 correspond to the nucleotides starting at the 5' end. There are binding sites for two iron atoms with four amino acids participating in the binding of each iron. The amino acids at positions Asp80, Tyr112, Tyr209, and His273 are required for coordination with one iron, and amino acids at positions Asp415, Tyr455, Tyr548, and His617 bind the other. There are two glycosylation sites at positions Asn157 and Asn498. The numbers refer to the deduced amino acid sequence. There are 25 amino acids per line of protein sequence (starting at nucleotide 18).

The nucleotide sequence analysis was performed on cDNA isolated from a human prostate cDNA library. The prostate cDNA library yielded a 2,140 bp cDNA which contained the complete 5' end including the untranslated portion and the signal sequence. The 3' end including the three amino acids at the carboxy terminal and the untranslated region were obtained as a 208 bp cDNA from both a monocyte cDNA library and human prostate cDNA library.

The data in Fig. 2 displays the full length cDNA sequence of this invention. The complete sequence including the 5' untranslated region and signal peptide have not been reported. Further, the previously reported amino acid sequence varies from the deduced amino acid sequence sequence for hLF of this invention. The following TABLE 1 is a summary of the differences of the amino acid sequence of the present invention and those reported by Metz-Boutigue et al, Eur. J. Biochem., vol. 45, pp. 659-6 (1984). For the purpose of this table, the numbering of the amino acids will be initiated with methionine at the start of the signal peptide sequence as amino acid #1.

TABLE 1
COMPARISON OF AMINO ACID SEQUENCES
HUMAN LACTOFERRIN

Amino Acid Deduced from cDNA of hLF	Change	Metz-Boutique Sequence
# 30 Thr	Substitution	Ala
# 48 Arg	Substitution	Lys
# 141 Arg	Insertion	NONE
# 155 Phe	Substitution	Leu
# 156 Leu	Substitution	Phe
# 170 Ala	Insertion	NONE
# 204 Ser	Substitution	Leu
# 206 Gln	Substitution	Lys
# 209 Tyr	Substitution	Lys
# 321 Lys	Substitution	Phe
# 386 Glu	Substitution	Gln
# 392 Ser	Substitution	Trp
# 410 Asp	Substitution	Asn
# 411-412	Deletion	13 Amino acids in protein sequence not in deduced amino acid sequence from cDNA.
# 532 Gln	Substitution	Glu
# 695 Lys	Substitution	Arg

Fig. 3 is the expression of human lactoferrin protein from the complete hLF cDNA. The complete 2358 bp hLF cDNA was ligated to the eucaryotic expression vector, p91023(B) at the EcoR1 site downstream from the adenovirus major late promoter. This plasmid vector was provided by Genetics Institute (Cambridge, Massachusetts) and has been described in previous publications (Wong et al., Science 288, 810-815 (1985)). The hLF cDNA expression vector was transferred into COSM-6 monkey kidney cells using standard tissue culture transfection conditions (Wigler et al. (1979) "Transformation of Mammalian Cells with Genes from Procaryotes and Eucaryotes"; Cell, 16:777-785). These COS cells do not normally express lactoferrin. Forty-eight hours after transfection, the cells were harvested and crude cell extracts were prepared. Positive identification of the human lactoferrin was made by standard Western immunoblot analysis of the proteins expressed in the cell extracts, as

well as those secreted into the cell growth medium using a commercially available antibody directed against human lactoferrin (Sigma). Proteins which bound to the anti-lactoferrin antibody were detected using radio-iodine labelled Protein A which reacts with the antibody. The immunoblots were autoradiographed to identify the human lactoferrin protein. Fig. 3 is an autoradiographic film showing the human lactoferrin expressed in four cell extracts prepared from tissue culture cells which were transfected with the lactoferrin cDNA expression vector (lanes 5 to 8). Lanes 5 to 8 show that the transfected cells all contain human lactoferrin (marked with an arrow) which is immunoreactive with the antilactoferrin antibody and is the same molecular weight as human lactoferrin (M_r = 78,403 daltons). The control cells which were not transfected with the cDNA did not contain lactoferrin (lanes 3 and 4). Analysis of the growth medium showed that human lactoferrin was also secreted into the medium from transfected cells (lane 2) but not from control cells (lane 1).

In conclusion, the cDNA encodes a recombinant human lactoferrin protein which is similar to human lactoferrin protein isolated from milk as determined by molecular size comparisons and immunoreactivity with antihuman lactoferrin. Furthermore, the secretion signal peptide sequence is functional since the human lactoferrin is secreted into the growth medium of tissue culture cells which express the cDNA.

Fig. 4 is a schematic representation of the human lactoferrin protein precipitated after <u>in vitro</u> transcription and translation of the human lactoferrin cDNA. The 2140 bp cDNA was from the human prostate cDNA library and included the 5' untranslated region and the rest of the base pairs correlative to the cDNA sequence of Fig. 2 omitting the last 218 bp at the 3' terminus. The 2140 bp cDNA was ligated to the EcoR1 site of the plasmid vector $pGEM_4$ (commercially available from Promega Biotech., Madison, WI 53711-5305) downstream from the SP_6 promoter. The plasmid

construct was linearized at the 3' end of the hLF cDNA using the restriction enzyme Hinc II or XBAI. The linear DNA template was then transcribed in vitro using purified SP_6 RNA polymerase in the presence of ribonucleotides as described in the manufacturers protocol (Promega Corporation 1988/1989 Catalogue and Applications Guide). The resultant mRNA was translated using 100ng mRNA template and micrococcal nuclease treated rabbit reticulocyte lysate (as described by Promega) in the presence of 75uCi ^{35}S methionine (800 ci/mmol, Amersham). In vitro synthesized lactoferrin was immunoprecipitated by incubating 100ul aliquots of translation reaction with 10ug of rabbit anti-human lactoferrin IgG (Sigma Chemical Company, St. Louis, MO 63178) for 2 hours at 4°C in 50mM Tris, pH7.5/0.15M NaCl/0.05% Tween-20 (1P-buffer). The reaction volume was 200ul. Immunoreactive lactoferrin was precipitated after incubation for 1 hour with 50ug of Protein A sepharose (Pharmacia, Upsalla, Sweden). Immunoprecipitation was carried out by centrifugation for 5 minutes at 10,000g and the precipitate was washed 5 times with 4 volumes of 1P buffer. Total translation products and immunoprecipitates were then subjected to electrophoresis in denaturing 7.5% polyacrylamide gels. After fixing in 50% Methanol, the gels were incubated in En^3Hance (NEN, DuPont, Wilmington, DE 19801) for 1 hour and washed with distilled H_2O. The gel was then dried under vacuum and exposed to Kodak X-OMAT XAR film at -70°C.

Lane 1 shows ^{14}C protein molecular weight markers used to estimate the size of the translated proteins. Lane 2 is a negative control which shows that no ^{35}S labelled proteins are translated in this system when no mRNA is added to the translation mix. Lanes 3 and 4 show the total translation products obtained when lactoferrin mRNA is added after preparation from two separate DNA templates. The major protein band (marked with an arrow) is human lactoferrin. This is the only band detected when the translation products are immunoprecipitated with anti human lactoferrin before

applying the protein to the gel (lane 6). The measurement of molecular mass by SDS-PAGE does not correspond to exact molecular weight due to secondary protein structure. However, the values are shifted in a correlative manner in comparison to the control. Analysis of the size of the translated lactoferrin is shown in Fig. 4. The protein migrated at the expected molecular mass of human lactoferrin (about 78Kd). The major bands in lanes 3 and 4 which migrate higher than the 68Kd marker band in the control lane correspond to expected molecular mass of hLF protein on SDS-PAGE.

In addition to using the entire cDNA sequence and deduced amino acids sequence, a polypeptide of less than the entire protein can be of value. For instance, the region between amino acids 74-275 contains an iron binding domain which may be used without the rest of the protein for biologically available iron or the bacteriocidal/viracidal qualities.

The cDNA sequence has been confirmed by corroboration of the sequence from three sources. The hLF cDNA was shown to encode lactoferrin by expression of the cDNA in a eucaryotic expression system and detection of the expressed lactoferrin protein by Western immunoblot analysis using specific lactoferrin antibodies.

WHAT WE CLAIM IS: 10

1. A cDNA sequence coding for human lactoferrin protein, comprising the DNA sequence of Fig. 2.

2. A cDNA sequence coding for the functional equivalent of human lactoferrin comprising analogues of the cDNA sequence of Fig. 2.

3. A synthetic human lactoferrin comprising a product produced from the cDNA sequence of Fig. 2.

4. A synthetic functional equivalent of human lactoferrin comprising a product produced from analogues of the cDNA sequence of Fig. 2.

5. A method to produce synthetic human lactoferrin product comprising utilizing the cDNA sequence of Fig. 2.

6. A method to produce a synthetic functional equivalent of human lactoferrin comprising utilizing analogues of the cDNA sequence of Fig. 2.

7. A method to produce synthetic human lactoferrin and functional equivalents thereof of claims 5 and 6 utilizing a eucaryotic expression system.

8. A cDNA sequence comprising a portion of the cDNA of Fig. 2 coding for human lactoferrin protein including at least one of the iron binding domains with an Fe binding site.

9. A cDNA sequence of claim 8 wherein an analogue of a portion of the cDNA sequence codes for a functional equivalent of at least one iron binding domain with an Fe binding site.

10. A method comprising producing a portion of synthetic human lactoferrin of claim 8.

11. A method comprising producing a portion of synthetic functional equivalent of human lactoferrin of claim 9.

12. A synthetic human lactoferrin product comprising a portion of the synthetic human lactoferrin product produced from the cDNA sequence of Fig. 2 including at least one of the iron binding domains with an Fe binding site.

13. A synthetic human lactoferrin product comprising a synthetic functional equivalent of a portion of the human lactoferrin protein produced by an analogue of the cDNA sequence of Fig. 2 including at least one functional equivalent of the iron binding domains with a Fe binding site.

FIGURE 1

Scheme of Human Lactoferrin (hLF) cDNA

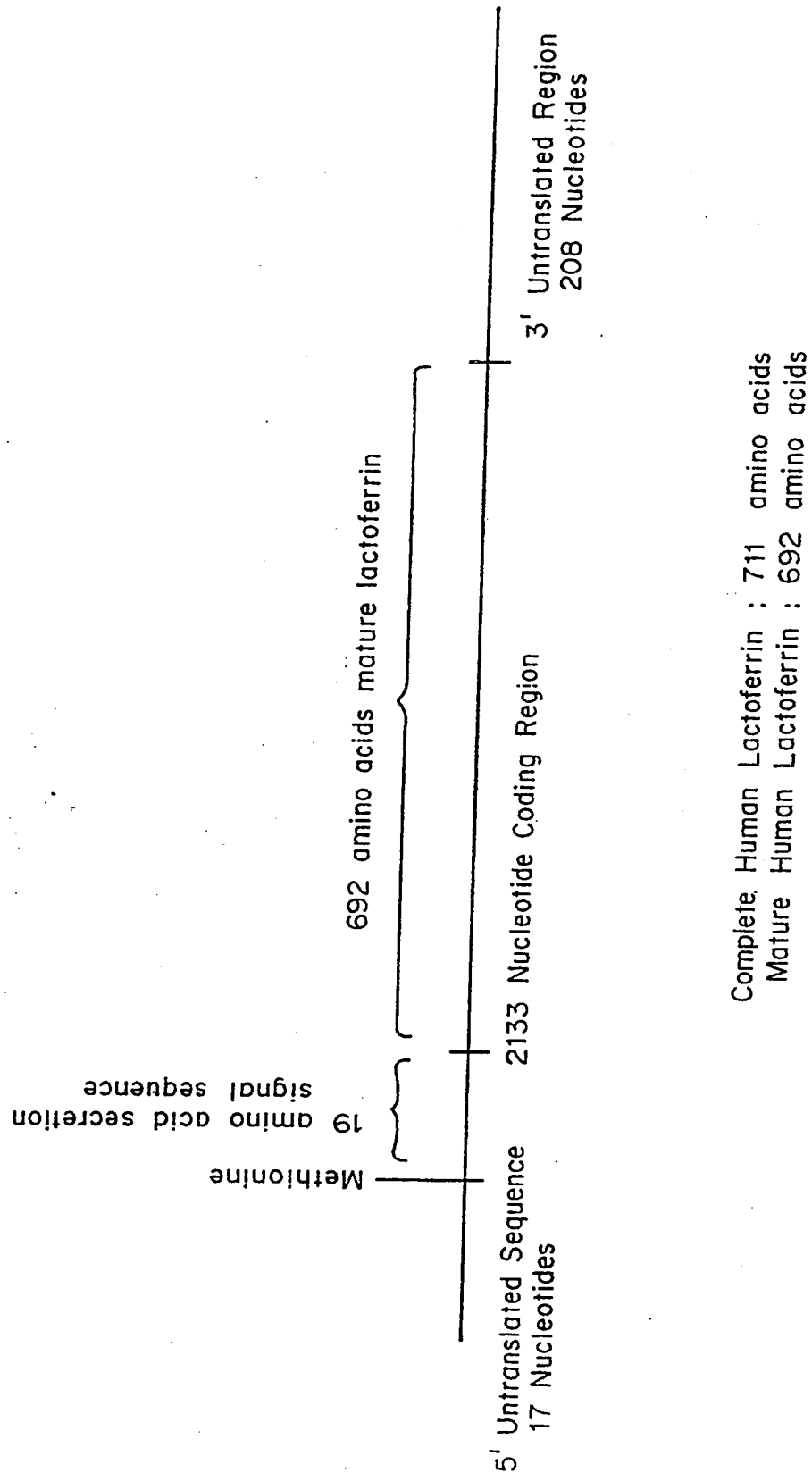

fig. 2

cDNA SEQUENCE AND AMINO ACID SEQUENCE
OF HUMAN LACTOFERRIN

```
                                                          1
                                                          GAATTCC GACCGCAGAC
 18
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  met lys leu val phe leu val leu leu phe leu gly ala leu gly leu
   1
 66
  TGT CTG GCT GGC CGT AGG AGA AGG AGT GTT CAG TGG TGC ACC GTA TCC
  cys leu ala gly arg arg arg arg ser val gln trp cys thr val ser
   17
114
  CAA CCC GAG GCC ACA AAA TGC TTC CAA TGG CAA AGG AAT ATG AGA AGA
  gln pro glu ala thr lys cys phe gln trp gln arg asn met arg arg
   33
162
  GTG CGT GGC CCT CCT GTC AGC TGC ATA AAG AGA GAC TCC CCC ATC CAG
  val arg gly pro pro val ser cys ile lys arg asp ser pro ile gln
   49
210
  TGT ATC CAG GCC ATT GCG GAA AAC AGG GCC GAT GCT GTG ACC CTT GAT
  cys ile gln ala ile ala glu asn arg ala asp ala val thr leu asp
   65
258
  GGT GGT TTC ATA TAC GAG GCA GGC CTG GCC CCC TAC AAA CTG CGA CCT
  gly gly phe ile tyr glu ala gly leu ala pro tyr lys leu arg pro
   81
306
  GTA GCG GCG GAA GTC TAC GGG ACC GAA AGA CAG CCA CGA ACT CAC TAT
  val ala ala glu val tyr gly thr glu arg gln pro arg thr his tyr
   97
354
  TAT GCC GTG GCT GTG GTG AAG AAG GGC GGC AGC TTT CAG CTG AAC GAA
  tyr ala val ala val val lys lys gly gly ser phe gln leu asn glu
  113
402
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  129
450
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  trp asn val pro ile gly thr leu arg pro phe leu asn trp thr gly
  145
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  193
 642
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  tyr phe ser tyr ser gly ala phe lys cys leu arg asp gly ala gly
  209
 690
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  asp val ala phe ile arg glu ser thr val phe glu asp leu ser asp
  225
 738
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  glu ala glu arg asp glu tyr glu leu leu cys pro asp asn thr arg
  241
 786
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  lys pro val asp lys phe lys asp cys his leu ala arg val pro ser
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1170
   AGC GAA GGC AGC GTG ACC TGC TCC TCG GCC TCC ACC ACA GAG GAC TGC
   ser glu gly ser val thr cys ser ser ala ser thr thr glu asp cys
   385
1218
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   ile ala leu val leu lys gly glu ala asp ala met ser leu asp gly
   401
1266
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   gly tyr val tyr thr ala gly lys cys gly leu val pro val leu ala
   417
1314
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   glu asn tyr lys ser gln gln ser ser asp pro asp pro asn cys val
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1362
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   asp thr ser leu thr trp asn ser val lys gly lys lys ser cys his
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1458
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   thr ala val asp arg thr ala gly trp asn ile pro met gly leu leu
   481
1506
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   497
1554
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   cys ala pro gly ser asp pro arg ser asn leu cys ala leu cys ile
   513
1602
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1842
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1890
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1938
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   641
1986
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2300
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```

fig. 3

EXPRESSION OF RECOMBINANT HUMAN LACTOFERRIN PROTEIN FROM hLF cDNA (COS M6 – CELLS)

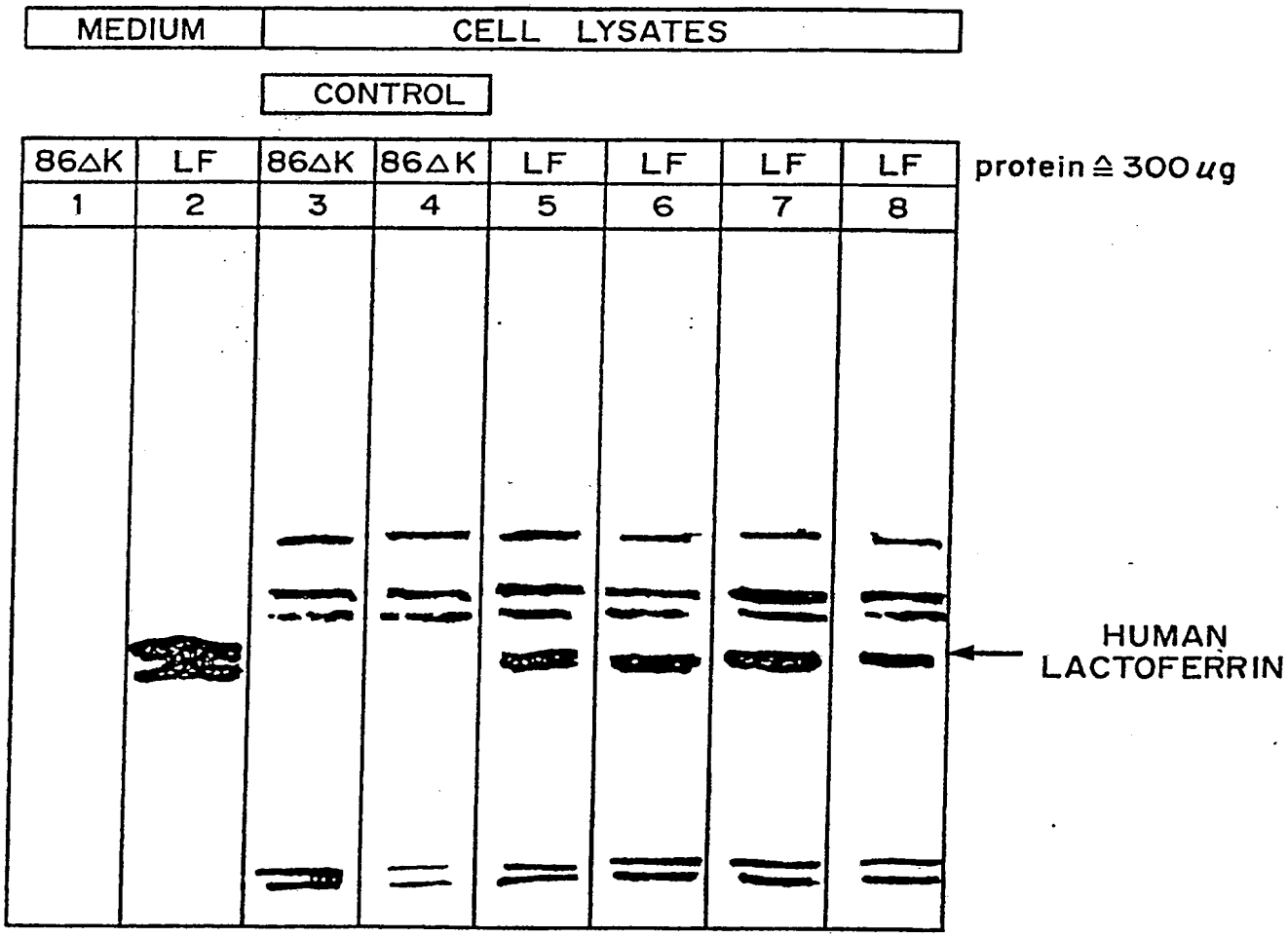
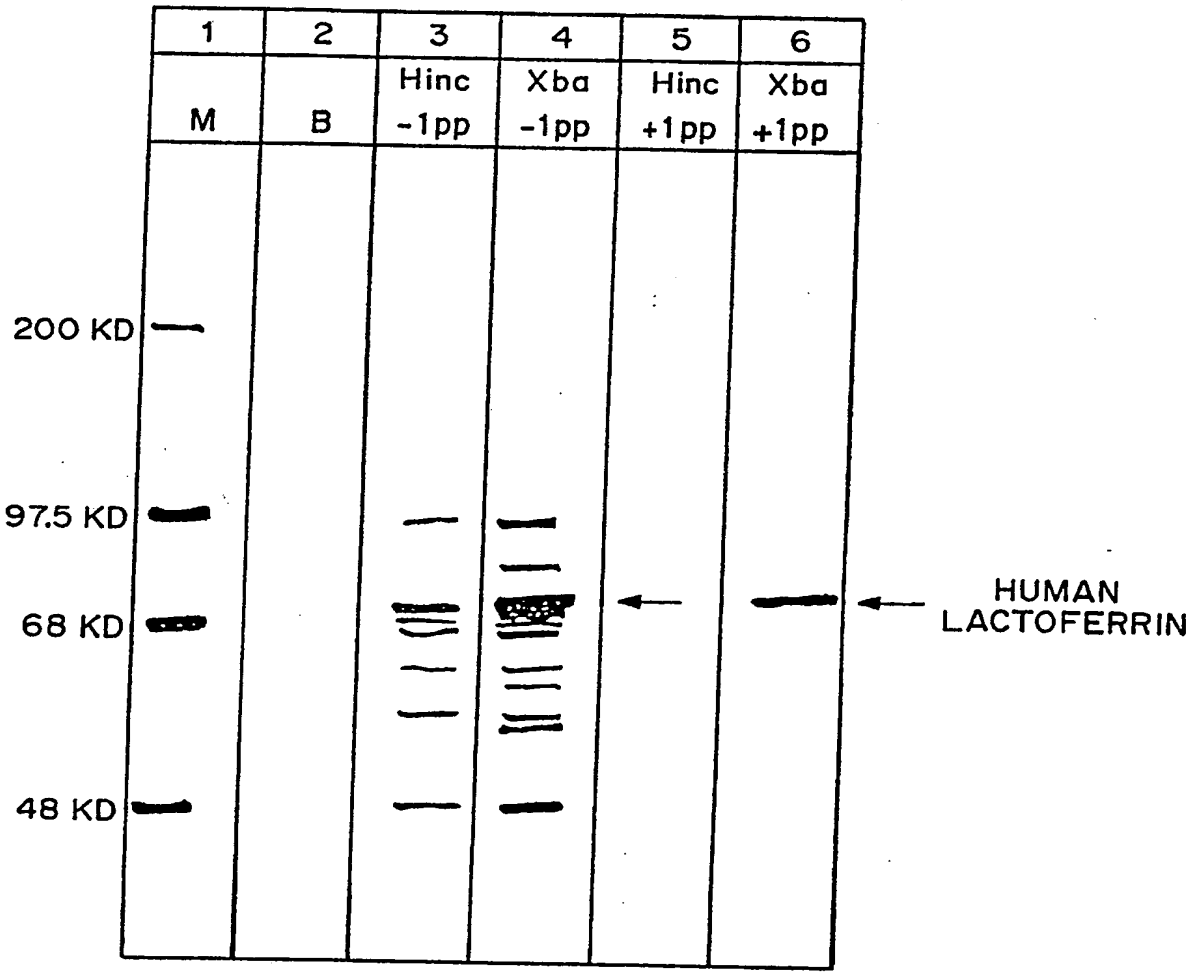


fig. 4

IN VITRO TRANSLATION 2140 bP hLF SEQUENCE



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/02356

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/12

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N, C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Blood, volume 79, no. 4, 1987, Grune & Stratton, Inc., (New York, US), T.A. Rado et al.: "Isolation of lactoferrin cDNA from a human myeloid library and expression of mRNA during normal and leukemic myelopoiesis", pages 989-993 see the whole article cited in the application	1-6,8,9
Y	European Journal of Biochemistry, volume 145, 1984, FEBS, (Berlin, DE), M.-H. Metz-Boutigue et al.: "Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins", pages 659-676 see the whole article cited in the application	1-6,8,9
X	--	12,13
P,X	American Dairy Science Association and American Society of Animal Science Combined Annual Meeting, ./.	1,2,8,9

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21st August 1990	04. 10. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	R.J. Eernisse

Form PCT/ISA/210 (second sheet) (January 1985)

International Application No PCT/US 90/02356

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	31 July - 4 August 1989, Lexington, Kentucky, Abstracts, Journal of Dairy Science, volume 72, suppl. 1, (US), L. Qianwa et al.: "Screening and cloning a cDNA coding for lactoferrin from human mammary gland", page 154, abstract 381, see the whole article	
A	-- The Journal of Biological Chemistry, volume 262, no. 21, 25 July 1987, (US), B.T. Pentecost et al.: "Lactotransferrin is the major estrogen inducible protein of mouse uterine secretions", pages 10134-10139 see the whole article	2

Form PCT/ISA 210(extra sheet) (January 1985)